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REVIEW ARTICLE

Incorporating germination-induction into decontamination strategies for bacterial spores

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Summary

Bacterial spores resist environmental extremes and protect key spore macromolecules until more supportive conditions arise. Spores germinate upon sensing specific molecules, such as nutrients. Germination is regulated by specialized mechanisms or structural features of the spore that limit contact with germinants and enzymes that regulate germination. Importantly, germination renders spores more susceptible to inactivating processes such as heat, desiccation, and ultraviolet radiation, to which they are normally refractory. Thus, germination can be intentionally induced through a process called germination-induction and subsequent treatment of these germinated spores with common disinfectants or gentle heat will inactivate them. However, while the principle of germination-induction has been shown effective in the laboratory, this strategy has not yet been fully implemented in real-word scenarios. Here, we briefly review the mechanisms of bacterial spore germination and discuss the evolution of germination-induction as a decontamination strategy. Finally, we examine progress towards implementing germination-induction in three contexts: biodefense, hospital settings and food manufacture.

Significance and Impact: This article reviews implementation of germination-induction as part of a decontamination strategy for the cleanup of bacterial spores. To our knowledge this is the first time that germination-induction studies have been reviewed in this context. This article will provide a resource which summarizes the mechanisms of germination in *Clostridia* and *Bacillus* species, challenges and successes in germination-induction, and potential areas where this strategy may be implemented.

Introduction

In the mid-19th century, Perty and Pasteur published the first observations of bacterial spores. Cohn built upon this work by witnessing the germination of spores under the microscope (Morrison and Rettger 1930). The advent of molecular genetics in the mid-20th century facilitated the identification of bacterial genes involved in germination. At the same time, germinants, germination enhancers, and germination inhibitors were also being identified.

Members of the *Clostridia* and *Bacillus* genera are the most-studied bacterial species that form spores. These species include both nonpathogenic species (such as *Bacillus subtilis*) and pathogenic species (such as *Bacillus anthracis, Clostridium difficile, Clostridium perfringens, Clostridium botulinum* and *Bacillus cereus*). *Bacillus anthracis* is the causative agent of anthrax (Dixon *et al.* 1999), while *C. botulinum, C. perfringens* and *B. cereus* cause food poisoning, and *C. difficile* is a major cause of hospital-acquired and wound-associated infections (Tang *et al.* 2014). Eradication of spores typically relies on strong disinfectants or toxic chemicals, including 10% bleach solutions and fumigation with chlorine dioxide. These strategies can be challenging to implement over large areas or on porous surfaces, such as soil. Additionally, the decontaminants may pose a risk to the environment, first responders and remediation teams.

Germination-induction has the potential to enhance decontamination because germinated spores are demonstrably less pathogenic and more susceptible to common disinfectants and other methods of killing. Given the relative ease of inducing germination, this strategy is applicable for multiple settings, each possessing their own unique challenges. Here, we briefly review the known mechanisms of bacterial spore germination and the potential for germination-induction to enhance decontamination efforts in biodefense scenarios, food manufacturing and the hospital environment.

Anatomy of the spore

The structure of the spore allows it to evade severe environmental conditions such as heat, cold, desiccation and ultraviolet (UV) radiation (Driks 2002, 2003). Spores contain an inner core that is surrounded by the inner membrane, and then the peptidoglycan cell wall and cortex (Driks 2002; Giorno *et al.* 2007, 2009). The cortex is surrounded by the outer membrane, then the coat, spore crust, and finally, for some species, the exosporium (Fig. 1).

The bacterial genome, as well as tRNAs, ribosomes, and most enzymes within the spore, are contained within the spore core. Small, acid-soluble proteins (SASPs) of the α/β type bind the genomic DNA (Setlow 1975, 1978; Setlow and Setlow 1979). SASPs are highly conserved in both *Clostridia* and *Bacillus* species but are not found in other species that do not sporulate (Setlow *et al.* 2006). Mutants of *B. subtilis* that lack functional α/β SASPs form spores that are less resistant to heat and UV radiation when compared to wild-type spores (Tovar-Rojo and Setlow 1991; Setlow and Setlow 1995). Thus, the SASPs play a key role in allowing the spore to protect its genome and survive. The spore core contains a high concentration of pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA). DPA is a compound found exclusively in spores and chelates Ca^{2+} yielding Ca-DPA. The high level of Ca-DPA lowers the water content within the spore

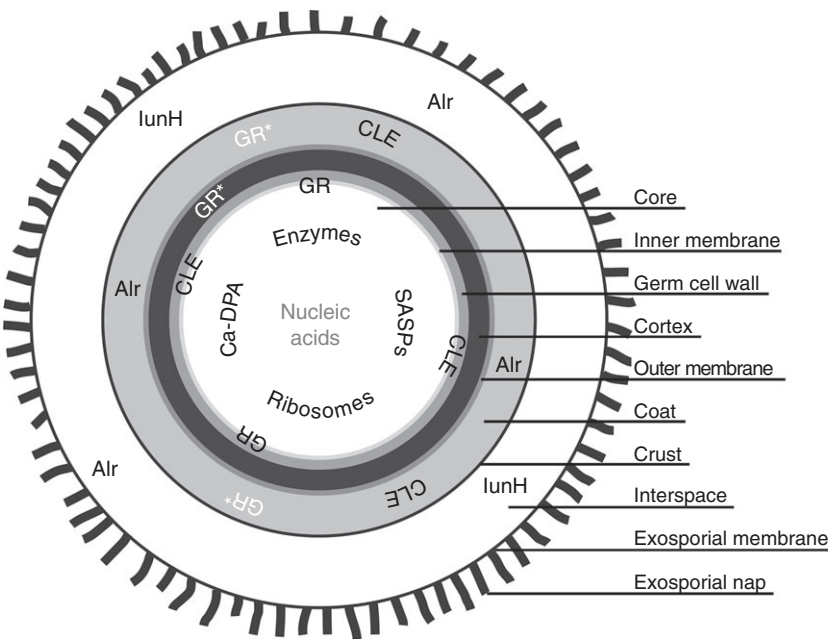

Figure 1 Graphical representation of a bacterial spore. Not all bacterial spores possess all of the described attributes, however, this figure depicts the major features of a bacterial spore (not drawn to scale, as different species have different structural proportions). The core contains nucleic acids, small acid-soluble proteins (SASPs), enzymes, ribosomes and Ca-DPA. The inner membrane contains the germinant receptors (GR) as well as the cortex lytic enzyme (CLE) SleB in *Bacillus* spores. SleB has also been localized to the integument fractions of spores (Chirakkal *et al.* 2002). The germ cell wall becomes the vegetative cell wall in the bacterial cell after germination initiation and bacillary outgrowth. The cortex layer is made of peptidoglycan and is surrounded by the outer membrane. The CLEs SleC and SleM are found near the outer edge of the cortex in some *Clostridium* species. The coat is a complex layer of multiple redundant proteins designed to protect the spore core from environmental insults such as desiccation. In *Bacillus* spores, the coat has been shown to contain alanine racemase (Alr) as well as the CLE CwlJ. Germinant receptors (GR*) in *Clostridium* spores may be located in the cortex layers or the spore coat. The crust is the outermost layer in some species of spores not containing an exosporium. The exosporium is a loose-fitting membrane surrounding the spore. The interspace between the spore and the exosporium contains multiple proteins including Alr and inosine hydrolase (IunH). In many species there is a 'hair-like' nap protruding from the exosporial membrane. BclA is a major protein comprising this nap in both *Bacillus* and *Clostridium* spores.

core facilitating spore dehydration and protects core proteins and DNA against damage. Ca-DPA accumulation also promotes resistance of the spore against both wet and dry heat, as well as hydrogen peroxide (Setlow *et al.* 2006). However, Ca-DPA is not required for spore formation (Setlow *et al.* 2006).

Moving outward from the spore core is the inner membrane. The inner membrane acts as a permeability barrier, and reduces access to the core by molecules that could potentially damage the DNA or other essential molecules held within the core (Cortezzo *et al.* 2004). In *Bacillus* spores, germinant receptors (GR) localize to the inner membrane, thus allowing a platform for germinant sensing that is protected by the outer layers of the spore (Hudson *et al.* 2001; Griffiths *et al.* 2011) (Fig. 1).

Unlike the inner membrane, the function of the outer membrane remains in question. This structure is located under the spore coat but appears to have no significant contribution to spore resistance properties (Nicholson *et al.* 2000; Leggett *et al.* 2012). This outer membrane may play a role in size exclusion (Rode *et al.* 1962; Setlow and Setlow 1980; Swerdlow *et al.* 1981; Gerhardt *et al.* 1982). In *Bacillus megaterium,* the fractionated outer membrane contains a significant portion of the spore cytochrome content as well as enzymes important in the electron transport chain such as NADH oxidase, dehydrogenase, cytochrome c reductase and NADPH dehydrogenase (Crafts-Lighty and Ellar 1980).

Between the inner and outer membranes are the germ cell wall and the cortex. Both the germ cell wall and cortex are composed of peptidoglycan. There are structural differences between the peptidoglycan in each of these layers such as differences in the levels of peptide crosslinking (Meador-Parton and Popham 2000). Cortex peptidoglycan contains muramic lactams, while germ cell wall peptidoglycan does not (Warth and Strominger 1969; Atrih and Foster 1999). This structural difference is a specificity determinant which allows cortex lytic enzymes (CLEs) to only degrade the cortex peptidoglycan (Atrih *et al.* 1996; Popham *et al.* 1996). The structure of peptidoglycan in the cortex is conserved amongst many *Clostridia* and *Bacillus* species, with some small modifications between species. Similar to the SASPs and inner membrane, the cortex contributes to the ability of spores to resist heat inactivation (Atrih and Foster 2001).

The spore coat is a multi-layered proteinaceous structure that lies either at the outer rim of the spore, or in species that possess an exosporium, just underneath the exosporium. The coat is a flexible structure with folds (Driks 2003). The spore coat slightly unfolds and allows the inner portions of the bacterial cell, especially the core, to swell when humidity increases (Sahin *et al.* 2012). Myriad proteins make up the spore coat and differ between species more than any other proteins essential for sporulation (Abhyankar *et al.* 2013; Driks and Eichenberger 2016). Importantly, the spore coat offers a layer of protection to the spore that allows smaller molecules, such as germinants, to pass to the inner membrane, while shielding the spore from other molecules in the environment. Different approaches have estimated the molecular weights excluded by the spore coat and other structures in *Bacillus* spores to range from approximately 2–160 kDa (Gerhardt and Black 1961; Nishihara *et al.* 1989; Henriques and Moran 2007; Knudsen *et al.* 2015). More recently, another layer outside of the spore coat has been identified. This layer, deemed the spore crust, is composed of glycoproteins, but is distinct from the exosporium. For some bacterial spores that lack an exosporium, this is the outermost layer of the spore that adds further protection from the environment as seen in spores of *B. subtilis* (McKenney *et al.* 2010).

Finally, some species of bacterial spores possess an exosporium as the outermost layer. The exosporium is a complex, proteinaceous structure that extends outward from the cell in fine hair-like projections (Gerhardt and Ribi 1964; Gerhardt 1967). The exact purpose of the exosporium is unclear, however, it has proposed functions in germination and interaction with the immune system in bacterial species that require the infection of a host to propagate (Bozue *et al.* 2015). In the case of *B. anthracis*, the exosporium *Bacillus* collagen-like protein of *anthracis* (BclA) contributes to exosporium hydrophobicity (Brahmbhatt *et al.* 2007). The exosporium may play a role in the protection of spores against macrophage killing (Kang *et al.* 2005) but does not significantly impact virulence in murine models (Bozue *et al.* 2007). Additionally, the BclA protein inhibits the adherence of spores to nonprofessional phagocytes and preferentially directs the interaction of spores with professional phagocytes (Bozue *et al.* 2007). Some *Clostridia* species are also known to have an exosporium, including *C. difficile* (Hodgkiss *et al.* 1967). Recently, the CdeC protein was identified as being required for exosporium formation in *C. difficile* (Barra-Carrasco *et al.* 2013). *Clostridium difficile* also expresses three collagen-like proteins, and one of them, BclA1, localizes to the exosporium (Pizarro-Guajardo *et al.* 2014). *Clostridium difficile* mutants containing an inactivated *bclA1* gene germinate more quickly and are less infectious in mice and hamsters (Phetcharaburanin *et al.* 2014).

Germination

Germination is an essential step in the lifecycle of bacterial spores (Moir *et al.* 2002; Moir 2003). Germination

occurs when GRs detect germinants in the environment. While *Bacillus* species contain GRs on the inner membrane, some species of *Clostridium*, such as *C. difficile*, express pseudoproteases that act as GRs in the spore coat and cortex (Fig. 1; Shimamoto *et al.* 2001; Adams *et al.* 2013; Francis *et al.* 2013). In some cases, germinants may act as sole germinants and some may act in concert as a co-germinant. In the case of *B. anthracis*, L-alanine can act as a sole-germinant, but only at high concentrations that may not be biologically relevant. However, the presence of a co-germinant such as inosine dramatically lowers the threshold levels of L-alanine required to trigger germination. The exact combination or concentration of germinants that is required to result in germination depends on the bacterial species, and different germinant combinations may prompt germination of different bacterial species (Wax and Freese 1968). For example, the GerK and GerL receptors of *B. anthracis* together detect L-alanine, while separately they detect proline and methionine or serine and valine, respectively (Fisher and Hanna 2005). Both *B. cereus* and the insect pathogen *Bacillus thuringiensis* may use inosine as a sole germinant (Clements and Moir 1998; Barlass *et al.* 2002; Liang *et al.* 2008), whereas inosine can only function as a co-germinant for *B. anthracis* (Fisher and Hanna 2005). Many germinants are molecules associated with environments favourable for bacterial growth and replication. For example, bile salts in the gut, calcium and amino acids act as co-germinants for the gastrointestinal pathogen *C. difficile* (Wilson *et al.* 1982; Wilson 1983; Wheeldon *et al.* 2011; Kochan *et al.* 2017). Other known germinants of bacterial spores include various amino acids, sugars, potassium ions, purine nucleosides, high concentrations of Ca-DPA, amphiphiles such as dodecylamine, and high hydrostatic pressure (HHP; Table 1).

Other environmental conditions, such as temperature and pH, may also affect germination. The optimal ranges for these variables are dependent upon the bacterial species. For example, *C. botulinum* spores preferentially germinate at 32·5°C (Grecz and Arvay 1982), while *B. anthracis* spores germinate optimally at 22°C (Titball and Manchee 1987). Importantly for the laboratory, the tendency for spores of *Bacillus* species to germinate greatly increases after short exposure to 65–80°C heat (Keynan *et al.* 1964). Thus, investigators typically heat activate *Bacillus* spores before performing germination experiments in the laboratory. Similar to germinants, the optimal temperature and pH ranges in which spores germinate may reflect environmental conditions that promote growth and replication of a given species.

Structural features of the spore alter germination kinetics and the ability of spores to germinate. For example, transposon mutants of *B. cereus* that do not express full length YwdL, a protein involved in exosporium assembly, do not germinate in the presence of Ca-DPA, a known *B. cereus* germinant (Terry *et al.* 2011). Additionally, the coats of many *Bacillus* and *Clostridium* spores contain the enzyme alanine racemase, which converts L-alanine to D-alanine and inhibits L-alanine germination (Todd *et al.* 2003; Redmond *et al.* 2004; Shrestha *et al.* 2017). Thus, one function of alanine racemase is to protect the spore from spontaneous germination due to trace amounts of L-alanine in the environment or the mother-cell during sporulation (Stewart and Halvorson 1953; Yasuda *et al.* 1993; Chesnokova *et al.* 2009). Nucleoside hydrolases are also present in the spore and act to reduce the efficiency

Table 1 Commonly studied species of spore forming bacteria and their germinants. High-hydrostatic pressure is also a germinant for these spore species, with differing levels of pressure leading to either germination or killing of the spores depending on the species. Ca-DPA is a shared germinant of the listed *Clostridium* (*C. difficile* spores are the exception; Wang *et al.* 2015) and *Bacillus* species. All of these bacteria express alanine racemase, but it does not appreciably affect the germination of *C. botulinum*

Bacteria	Germinants and co-germinants	Reference
Bacillus subtilis	L-valine and L-ala, L-asparagine, D-glucose, D-fructose, K^+, dodecylamine	Pierce *et al.* (2008)
Bacillus anthracis	L-alanine, inosine, L-valine, L-serine, Purines, L-proline, L-methionine, L-histidine	Fisher and Hanna (2005)
Bacillus cereus	L-alanine, inosine, L-glutamine, L-asparagine, glucose, fructose, K^+	Barlass *et al.* (2002); Hornstra *et al.* (2005)
Bacillus thuringiensis	L-alanine, inosine	Yan *et al.* (2007)
Clostridium difficile	Glycine, L-alanine, L-cysteine, L-noraline, L-2-aminobutyric acid, L-phenylanine, L-arginine, bile acid, Ca^{2+}	Bhattacharjee *et al.* (2016); Kochan *et al.* (2017)
Clostridium perfringens	L-asparagine, L-glutamine, L-cysteine, L-threonine, L-serine, L-alanine, L-valine, KI, KBr, dodecylamine	Paredes-Sabja *et al.* (2008); Bhattacharjee *et al.* (2016)
Clostridium botulinum	L-alanine, L-cysteine, L-serine, L-lactate, L-phenylalanine, L-methionine	Alberto *et al.* (2003); Brunt *et al.* (2014); Bhattacharjee *et al.* (2016); Brunt *et al.* (2016)

of nucleoside germinants by converting them to a form that does not induce germination (Redmond *et al.* 2004; Liang *et al.* 2008). As discussed by Liang *et al.* regarding the inosine hydrolase in *B. thuringiensis*, nucleoside hydrolases function at optimal pHs, suggesting that environmental pH could affect germination efficiency.

Following germinant detection, there is a lag period lasting from minutes to greater than 24 h (depending on the bacterial species) until germination begins and leads to irreversible commitment of spores to germinate. Germination is then described as three separate phases: commitment, Stage I, and Stage II. A variety of factors, such as the presence of particular CLEs, levels of GRs, and concentrations of germinants influence the timing of commitment (Yi and Setlow 2010; Chen *et al.* 2014). Once commitment to germination occurs in *Bacillus* and some *Clostridium* species, monovalent cations are released. As germination progresses to Stage I, Ca-DPA is released and hydration of the core begins (Powell and Strange 1953; Swerdlow *et al.* 1981; Cowan *et al.* 2004). Then in Stage II, CLEs break down the peptidoglycan in the cortex (Makino and Moriyama 2002), and the core becomes fully hydrated. Notably, this sequence differs in *C. difficile* spores, with cortex hydrolysis occurring before Ca-DPA release (Francis *et al.* 2015). Species of spore-forming bacteria typically have multiple CLEs, which have different biochemical activities, allowing the spore to quickly and efficiently degrade its peptidoglycan upon germination. CLEs present in the ungerminated spore, such as CwlJ and SleB in *Bacillus* and some *Clostridium* spores, are essential germination machinery (Heffron *et al.* 2010, 2011). Additionally, during germination, the bacterial membrane becomes more fluid (Stewart *et al.* 1980; Cowan *et al.* 2004) and the spore core releases cations (Swerdlow *et al.* 1981). Finally, after breakdown of the cortex, germination is completed, and the germinated spore begins outgrowth as a replicating vegetative cell.

Spore populations may be heterogeneous, with some spores germinating after others. Indeed, in species of *Bacillus* and *Clostridium*, the dormancy of a small subset of spores is prolonged, a condition known as superdormancy (Gould *et al.* 1968). Superdormant spores require increased heat activation and have higher wet-heat resistance (Ghosh and Setlow 2010). Superdormant *Bacillus* spores germinate with kinetics similar to those of other spores after exposure to Ca-DPA or dodecylamine (Ghosh and Setlow 2010). Additionally, treatment with peptidoglycan fragments, bryostatin, or high pressure (≥5000 atm) induces germination of superdormant spores (Wei *et al.* 2010). Expression of GRs and conditions at the time of sporulation influence the probability of a given spore becoming superdormant (Ghosh and Setlow 2009). While fewer studies have been performed on the superdormancy phenomenon in *Clostridia* species, recent work suggests heterogeneity within *C. perfringens* spore populations (Wang *et al.* 2011, 2012). This heterogeneity may reflect the superdormancy of some spores within the population.

Quorum sensing has the potential to regulate superdormancy of spores. Quorum sensing in this context indicates that a given spore undergoing germination may trigger other spores to germinate. While this has been observed in *C. botulinum* (Zhao *et al.* 2006) the potential for quorum sensing to influence superdormancy in other species remains under investigation. In *Bacillus*, an analysis of thousands of individual spores undergoing germination revealed that the proximity of an ungerminated spore to other spores undergoing germination dictated the likelihood of the dormant spore to germinate; however, this was not dependent on the known germinants Ca-DPA or L-alanine (Zhang *et al.* 2011). Together these findings suggested that an unidentified quorum-sensing mechanism may be involved. Given the numerous factors that promote both sporulation and germination, it is likely that quorum sensing may synergize with other signals to determine the dormancy state of the spore.

Germination-induction and decontamination

Given the robust and resistant structure of bacterial spores, decontamination is a major challenge. One strategy is to induce germination. Upon transitioning to the vegetative form, bacteria are more susceptible to common disinfectants and methods of killing. This strategy was first devised by Stuy (1956). Inducing germination of *B. thuringiensis* or *B. anthracis* with L-alanine and inosine resulted in increased susceptibility to bleach, hydrogen peroxide, UV radiation, peracetic acid or formaldehyde (Omotade *et al.* 2014; Celebi *et al.* 2016). Whereas the former experiments were conducted in liquid suspension in the laboratory, the efficacy of germination-induction has also been demonstrated more recently on various surfaces (Mott *et al.* 2017). Importantly, the presence of other substances on a surface or in suspension can decrease the effectiveness of this strategy when used with particular disinfectants. For example, the germinants L-alanine and inosine decrease the effectiveness of bleach if they remain present after germination is induced in *B. anthracis* and *B. thuringiensis* (Omotade *et al.* 2014). Additionally, serum and other organic debris inactivate bleach, and salts decrease the activity of glutaraldehyde (Sagripanti and Bonifacino 1997). Similarly, microbicidal effects of UV radiation can also be reduced by the presence of solutes or other organic debris (Mansor *et al.* 2014; Omotade *et al.* 2014). However, other disinfectants,

such as hydrogen peroxide, are less affected by the presence of germinants or other molecules when germination-induction is implemented (Omotade *et al.* 2014; Mott *et al.* 2017). The efficacy of germination-induction has also been demonstrated for use with *Clostridia* spores in combination with commonly used disinfectants and high pressure treatments (Kalchayanand *et al.* 2004; Udompijitkul *et al.* 2013).

Germination-induction is an attractive option for decontamination in multiple contexts due to the difficulty of eradicating spores. For wide-area decontamination, current strategies are either not completely effective at eradicating spores or involve the use of hazardous chemicals or physical treatments, such as fumigation with chlorine dioxide, paraformaldehyde or vaporized hydrogen peroxide. These strategies pose a danger to first-responders and other personnel involved in decontamination as well as potentially having long-term environmental consequences. For medical or food applications, germination-induction provides an additional mechanism of inactivation to decontaminate products or surfaces that come into close proximity with, are ingested by, or are transplanted into humans.

Another potential strategy to eliminate bacterial spores is treatment with bacteriophages (Schuch *et al.* 2002). Indeed, it is unlikely that any bacteriophages would successfully kill dormant spores, however, it may be possible to identify a phage that could infect and kill a germinating spore. The identification of phages that can successfully eliminate spores or even successfully inject their DNA into bacterial spores is in progress, but germination-induction could be successfully paired with phage treatment. The investigation of germination-inducing or -enhancing molecules (i.e., other than known germinants) that could be used in decontamination efforts is ongoing. One such compound is D-cycloserine, which increases the effectiveness of L-alanine induced germination by the inhibition of alanine racemase activity and also enhances the efficacy of decontamination efforts of *B. anthracis* through its antibiotic activity (Gould 1966; Omotade *et al.* 2013). A similar strategy was used to enhance inactivation of four *Clostridia* species in food by adding a known antimicrobial (Kalchayanand *et al.* 2004).

To be effective, germination-induction strategies must not result in bacterial replication or a secondary sporulation event. Control can be exercised with optimized concentrations of specific germinants or compounds in lieu of simply treating spores with concentrated or complex nutrient solutions that may also promote bacillary outgrowth and subsequent sporulation. Solutions of L-alanine and inosine induce germination of *B. anthracis* and *B. thuringiensis* and render the spores susceptible to secondary disinfectants, but these solutions are not complex enough to allow for outgrowth and bacterial replication (Omotade *et al.* 2014). The identification of other compounds like D-cycloserine that enhance germination may improve the utility of deploying germination-induction as a decontamination strategy in the real world. Lastly, for some bacterial spore forming species, further work may be required to determine the natural germinants or co-germinants.

While germination-induction may reduce the number of viable bacteria after germination by up to several logs (without further treatment), a limitation of germination-induction is that there are typically a small number of ungerminated spores left behind. This population of spores may include super-dormant spores or other spores, which for various reasons, may not have germinated upon contact with the germination-induction solution. For this reason, it is proposed that germination-induction be coupled with a secondary method of disinfection. Germination-induction is an important strategy since it reduces the residual number of spores left after treatment with a disinfectant and could allow the usage of a milder disinfectant that poses fewer hazards to workers and the environment.

Biodefense applications

The potential for the accidental or intentional release of *B. anthracis* spores to cause damage was demonstrated in the aftermath of the Sverdlovsk incident in 1979 in the former Soviet Union and the 2001 anthrax letter attacks in the United States. In 1979, of 96 reported cases that are believed to have been predominantly inhalational infections, 64 people died (Meselson *et al.* 1994). In the 2001 attacks, five of the 22 people infected died, and the cost associated with containing and neutralizing spores released in affected facilities was over $320 million (Jernigan *et al.* 2002). Due to these incidents and the high potential for lethality after respiratory infection, *B. anthracis* remains the most studied pathogen for spore decontamination in biodefense contexts. Other spore forming bacterial pathogens of biodefense interest include *C. perfringens*, *C. difficile* and *B. cereus* for their potential to contaminate water and food sources, and *C. botulinum* and other *Clostridia* species for their ability to contaminate wounds, particularly in military health care settings. More recently, emerging strains of *B. cereus* which have acquired the toxins and attributes of *B. anthracis* have been shown to cause anthrax-like disease in humans and nonhuman primates (Hoffmaster *et al.* 2004, 2006; Avashia *et al.* 2007; Antonation *et al.* 2016).

Remediation efforts after the release of bacterial spores could require decontamination across large surface areas and a variety of materials. The decontamination of

outdoor areas poses a significant challenge due to the potential for environmental pollution if hazardous chemicals were used. The decontamination of Gruinard Island by the UK Ministry of Defence remains the best example of wide-area decontamination of bacterial spores (Manchee *et al.* 1981). This small island off of the Scottish coast was used for munitions testing involving small bombs containing *B. anthracis* spores during World War II and was considered a biological hazard area through the early 1980s. After decades of monitoring, large-scale decontamination efforts employing 5% formaldehyde in seawater were deemed successful (Manchee *et al.* 1994). However, the formaldehyde treatment and the pre-application of biocides required to encourage contact of the formaldehyde with soil could potentially have been lessened by including germination-induction strategies.

The porosity of soil renders it particularly difficult to decontaminate. In experiments using soil microcosms, induction of germination increased the sensitivity of *B. anthracis* to peracetic acid (Celebi *et al.* 2016). Similarly, the addition of L-alanine and inosine to soil microcosms contaminated with *B. anthracis* spores induced germination and the resultant vegetative bacteria did not survive after several weeks (Bishop 2014). This suggests that decontamination by merely inducing germination may be possible in more challenging environmental clean-up scenarios, such as in soil or near water sources, where the application of disinfectants may not be practical or possible. Germination-induction of *B. anthracis* spores deposited on nonporous surfaces resulted in a decrease in viability after an extended incubation, similar to when a disinfectant was applied to ungerminated spores (Mott *et al.* 2017). Thus, a large percentage of spores that have been induced to germinate will likely succumb to environmental conditions, such as sunlight or desiccation.

In addition to the decontamination of surfaces, the treatment of patients infected with spore forming pathogens after an accidental or intentional release could potentially be improved with germination-induction approaches. Survival was not improved in mice infected with a lethal dose of *B. anthracis* spores when the germinants L-alanine and inosine were introduced into the lungs postinfection (Cote *et al.* 2009). However, when mice were infected with pregerminated spores, the majority of the mice survived the challenge and exhibited no signs of illness (Cote *et al.* 2009). Conversely, when spores were exposed to D-alanine (to inhibit germination) prior to infection, mice were substantially more affected by the ensuing infection (McKevitt *et al.* 2007; Cote *et al.* 2009). Thus, administering germinants as a sole treatment to an infected host does not improve survival. By inducing germination as a decontamination plan after an accidental or intentional release of *B. anthracis,* people exposed to the site afterward could be less likely to become ill, even before treating the site with disinfectants. Recommendations suggest that *B. anthracis*-infected patients take a 60-day course of ciprofloxacin (Deziel *et al.* 2005; Hendricks *et al.* 2014). The longer antibiotic course provides a broader window for any remaining spores to germinate and be eliminated. It is unclear if germination-induction could be used to shorten the course of antibiotic treatment. This combination approach would have the potential to stem the development of antibiotic resistance and to improve therapies for difficult to treat infections. More work is required to understand potential benefits associated with manipulating the germination state of bacterial spores in a clinical setting.

Medical devices and the hospital environment

Clostridium difficile is the most prevalent spore forming bacterial pathogen in the hospital environment. The ability of *C. difficile* to form biofilms makes it difficult to eradicate, particularly on medical devices. This is in part due to the fact that *C. difficile* biofilms contain spores (Semenyuk *et al.* 2014) and the propensity for nosocomial *C. difficile* strains to acquire antibiotic resistance. Moreover, patients who are already receiving antibiotic treatment for other infections have increased probability for becoming infected with *C. difficile* due to changes in their gut microbiomes (de la Cochetiere *et al.* 2008). The exact mechanisms which promote the spread of *C. difficile* in hospital environments have not been completely identified, however, contaminated surfaces, transmission by health care personnel and asymptomatic carriers have been proposed (Rutala and Weber 2008).

Current strategies for decontamination of spore-forming pathogens involve disinfectants or exposure to intensified physical conditions. Effective forms of decontamination against *C. difficile* on medical devices prior to implantation include HHP, high temperature and bleach. In patients implanted with contaminated medical devices, the course of action is typically to administer long-course antibiotics with the option of removing the device, particularly if biofilms are thought to be present. Bleach is a recommended disinfectant for contaminated hospital surfaces (Rutala and Weber 2008), but as discussed earlier, its activity can be inhibited by factors such as organic debris (McDonnell and Russell 1999).

Inducing germination to supplement decontamination efforts during a *C. difficile* outbreak in a hospital environment would work similarly to what has been defined for biodefense applications after the release of *B. anthracis* spores. Indeed, germination-induction prior to secondary

disinfection increases the killing of *C. difficile* spores on a variety of hospital surfaces (Nerandzic and Donskey 2010). It is possible that specific induction of germination on implanted medical devices could improve antibiotic therapy when administered simultaneously. It was demonstrated that it is also possible to coat surfaces with triggers of spore germination and antimicrobials to eradicate vegetative bacteria (Fulmer and Wynne 2012). Further investigations will determine the effectiveness and appropriateness of employing germination-induction to control spore-forming pathogens in the hospital environment.

Food production and storage

Many spore forming bacterial pathogens such as *B. anthracis*, *B. cereus, Bacillus licheniformis, B. badis, Bacillus sporothermodurans*, *C. perfringens*, *C. botulinum, Bacillus amyloliquefaciens*, *Geobacillus steraothermophilus, Moorella thermoacetica* and *Thermoacnaeobacterium* species have been identified in contaminated food products (Gopal *et al.* 2015; Wells-Bennik *et al.* 2016). Current techniques for the eradication of micro-organisms in food include heat treatment, additives such as sodium benzoate, antimicrobial bacteriocins such as nisin, and HHP. However, these strategies are differentially effective for the eradication of spores. A major challenge for the food industry is to identify decontamination strategies that ensure proper eradication of spores without affecting the taste and quality of treated products.

The use of heat is one of the oldest techniques to control pathogenic bacteria in food. Sterilization involves exposure to high heat that is sufficient to kill all of the micro-organisms, while pasteurization uses lower heating temperatures to reduce the number of micro-organisms below a threshold safe for human consumption. For milk products, it is also possible to sterilize through the application of ultra-high temperatures. This involves heating the milk at 140–150°C for only a few seconds (Jay *et al.* 2005). Despite the effectiveness of these techniques to eliminate most micro-organisms, ungerminated spores are not typically eradicated by such heat treatments alone. Instead, coupling heat treatment with germination-induction could significantly decrease the number of viable spores of both *Clostridia* and *Bacillus* species.

Another strategy for food decontamination is the inclusion of additives, such as the antimicrobial peptide and bacteriocin nisin. Bacteriocins, such as nisin, are particularly attractive to both manufacturers and consumers because they are natural and do not result in detrimental effects as do other preservatives, such as sodium benzoate. Nisin is considered by the US Food and Drug Administration to be safe for consumption by humans. The bacterial species *Lactococcus lactis* naturally produces nisin, which was first developed as a food additive in the 1960s. Nisin can also be used to coat sheet metal in milk or food processing equipment to reduce the likelihood of biofilm formation (Gopal *et al.* 2015). While nisin is the most widely studied and used bacteriocin, there are a number of other bacteriocins being developed for use in food. These bacteriocins include lacticin 3147, enterocin AS-48, bificin C6165, plantaricin TF711, and thurincin H (Egan *et al.* 2016). For spores of *B. anthracis*, nisin disrupts the bacterial membrane by binding to lipid II after germination has begun, and inhibits the outgrowth of spores by inducing pore formation (Gut *et al.* 2008, 2011). Ultimately, nisin and other bacteriocins work by killing any spores upon germination, but they do not induce germination; therefore, germination-induction could improve this process. One danger in using decontamination strategies that rely on additives, such as nisin, is the acquisition and spread of resistance to such molecules. While reports of nisin resistance are low, acquisition of resistance has been documented (Jarvis 1967). Germination-induction paired with addition of nisin to food decreased the viability of several species of *Clostridia* spores (Kalchayanand *et al.* 2004).

The application of HHP is a third strategy for spore decontamination in food. While high pressures were known to kill bacteria since the late 19th century, HHP was not developed for use in the food industry until the 1990s (Demazeau and Rivalain 2011). HHP affects spore viability, especially when used in combination with other decontamination strategies such as heat or the addition of nisin. Lower pressure increases the germination efficiency of *B. subtilis* spores, but higher pressures can be combined with increased temperature for effective sterilization (Paidhungat *et al.* 2002). Similarly, *Clostridia* spores require high pressures of 400 MPa or more combined with heat of 70–110°C for inactivation (Kalchayanand *et al.* 2004). HHP represents an exciting future technology for spore decontamination in food that does not rely on additives, which is attractive to manufacturers who increasingly desire to cater to more conscious consumer habits regarding additives and preservatives.

Conclusion and outlook

Here, we have briefly reviewed the general mechanisms of bacterial spore germination and data supporting the use of germination-induction as a decontamination strategy in the contexts of biodefense, hospital environments and food production. Germination-induction offers a promising means of controlling bacterial spores without exposing workers or the environment to harsh conditions or chemicals. Future investigations to identify other

chemicals or compounds that may induce or enhance germination would provide additional means of germination-induction aside from known germinants that may be difficult or expensive to produce or deploy in real-world decontamination scenarios. Such investigations may also shed more light on questions surrounding spore biology and general germination mechanisms that are yet to be understood.

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Conflict of Interest

None.

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